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13. ABSTRACT *(Maximum 200 Words)* In this study, neuroprotective ganglioside derivatives are studied in an attempt to devise neuroprotective agents targeted to specific points in cell death pathways. GM1 ganglioside and several of its chemically modified derivatives are neuroprotective in a variety of neurotoxic models. Here, ganglioside functional groups required for neuroprotection and blood-brain barrier (BBB) permeance are determined. Cell death mechanisms are also defined, as are the mechanism(s) by which ganglioside derivatives intervene in the cell death process. In the third year, syntheses of various ganglioside derivatives have been accomplished. A significant delay in the proposed work was encountered, however, when a commercial reagent was acidic beyond specifications and, therefore, decomposed the lysoGM1 derivative that is also the starting material for many of the other derivatives. Negotiations are currently underway to determine company culpability and fair replacement. Fatty acid derivatives of GM1 have been tested in the SH-SY5Y cell culture system. LysoGM1, LIGA 20, N-butylGM1, N-MyristylGM1, and GM1 inner ester were all cytoprotective but at different effective concentrations. AsialoGM1 was not cytoprotective.

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INTRODUCTION

Gangliosides are naturally occurring acidic glycolipids that are enriched in neural tissue. They broadly protect different types of neurons *in vitro* (1;2) and preserve diverse neuronal (3-5) and behavioral (6;6) functions from a variety of neurotoxic insults *in vivo*. The fact that gangliosides appear to protect neurons from various neurotoxic insults by several different mechanisms makes them ideal candidates for, or models upon which to derive, therapeutic agents for Parkinson's disease and other neurodegenerative diseases with potentially diverse pathological mechanisms. Gangliosides are heterogeneous compounds differing in the composition of their lipophilic and hydrophilic moieties. It has been shown with the monosialoganglioside GM1 that the cytoprotective effects and blood-brain barrier permeance differ among the various molecular structures. This implies that the protective effects of gangliosides can be optimized by semisynthetically varying their lipophilic and hydrophilic moieties. Many neurotoxins work through mechanisms that involve mitochondrial electron transport chain inhibition and oxidative stress, proteasomal inhibition, or excitotoxicity. Gangliosides have been shown to be protective against a range of neurotoxins in various models. For example, GM1 protects dopaminergic neurons from MPTP (7;8) and MPP^+ (9) (a mitochondrial complex I inhibitor) even when administered after neurotoxin exposure (10). Gangliosides appear to be protective through several mechanisms. These minimally include their effects on growth factor production and receptor activation (11), signal transduction (12), membrane order and raft formation (13), calcium homeostasis (14), membrane trafficking, excitatory processes (15), altered enzyme activity (such as superoxide dismutase (16), catalase (17) or phosphatidylinositol 3-kinase (2)) and modified protein kinase C translocation (3). It is, of course, important to ultimately understand the mechanism of ganglioside neuroprotection. There is, however, neither an adequate understanding of ganglioside functional groups and required structure necessary to produce significant neuroprotection, nor a sufficient knowledge of the functional group alterations that allow ganglioside derivatives to effectively cross the BBB while maintaining cytoprotective ability. In fact, investigations into ganglioside protective mechanisms are daunting endeavors because of the apparent diverse modes of action. Thus, one approach to defining structure-function relationships in ganglioside neuroprotection is to first describe the structural requirements for neuroprotection and then to define the mechanisms of action.

One problem in using gangliosides for therapeutic purposes is that they are ionic and hydrophilic. This prevents them from efficiently crossing the BBB. An approach to overcoming the BBB limitation is to make gangliosides more lipophilic by derivatizing the parent compounds (semisynthetic gangliosides), and, presumably, making them better able to cross the BBB. The internal ester (lactone) of GM1 is chemically neutral (and, therefore, more lipophilic) and it is also neuroprotective *in vivo*,

often more potently than the parent GM1. (19), (20), (21), a (22). The apparent improvement, compared to the parent GM1, in the *in vivo* neuroprotection by ganglioside derivatives such as the internal ester or LIGA 20, may be due to either or both improved neuroprotective properties, and improved ability to cross the BBB.

This study examines ganglioside functional group derivatives that provide cytoprotection AND effectively cross the BBB; information that will provide a basis for future studies of neuroprotective mechanisms. This research studies the ability of ganglioside derivatives to be cytoprotective in *in vitro* models using the dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+) and the SH-SY5Y human neuroblastoma cell line. Derivatives determined to have therapeutic potential are tested *in vitro* for their ability to cross a brain capillary endothelial cell culture model of the BBB. Derivatives that are both cytoprotective and that effectively cross the *in vitro* BBB model will be tested *in vivo* for their ability to neuroprotect dopaminergic neurons in both chronic and acute neurotoxicity models using the MPP^+ precursor, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This research studies the hypothesis that "changes in ganglioside ceramide and/or oligosaccharide functional groups will improve neuroprotection through changes in cytoprotection and BBB transcytosis." This research will provide a basis to improve ganglioside neuroprotection in neurodegenerative diseases, e.g., Parkinson's disease and neurotoxin exposure.

BODY

Response to reviewer's comments on second annual report

(1) Objective #1 – *In progress, slow, but some success reported.....no data for quantitative yield or structural determination, such as chemical and mass spectrometry, as originally planned.....-* As described below, this project has been impeded by the substantial loss of lysoGM1 due to a commercial solvent that was acidic well beyond specifications. Considerable time and effort is required to produce this material from GM1. It is then used to synthesize the fatty acid molecular species used for *in vitro* testing and for the production of radiolabeled gangliosides. It is extremely unstable in an acidic environment. Negotiations are in progress with the manufacturer to recoup losses.

Still, several derivatives have been synthesized and characterized (Table 1). Gangliosides and their derivatives were characterized by chromatographic behavior compared to ganglioside standards, chemical reaction (e.g., hydrolysis of esters to the parent compounds), colorometric responses to specific sprays on HPTLC (e.g., resorcinol for sialic acid; orcinol for sugars, and ninhydrin for free amines) and by MS and MS/MS instrumental analyses. MS and MS/MS spectra of the derivatives were acquired by infusion of methanol solutions of each compound at 5 ul/min directly into the electrospray

ionization source of a Finnigan LCQ quadrupole ion trap mass spectrometer equipped with a Finnigan Xcalibur data system. Negative ion electrospray ionization was performed with 4,500 V source potential and MS/MS spectra were obtained using 33% collisional activation energy. The derivatives were identified by the $[M-H]^-$ ion and MS/MS fragment ions. Characterization of several inner esters of N-acylGM1 derivatives is in process.

Compound	Resorcinol	Orcinol	Ninhydrin	Retained by Ion Exchange	$[M-H]^-$ C18:0-d18:1	$[M-H]^-$ C18:0-d20:1
GM1	+	+	-	yes	1544	1572
GM1 inner ester	+	+	-	no	1526	1554
lysoGM1	+	+	+	untested	1261	1289
N-acetyl GM1 (LIGA4, C2)	+	+	-	yes	1320	1348
N-dichloroacetylGM1 (LIGA20, dichloroC2)	+	+	-	untested	1388	1416
GM1 gangliosidol	+(faint)	+	-	no	1530	1558
N-acetyl GM1 inner ester	+	+	-	no	1302	1330
AsialoGM1	-	+	-	no	1253	1281
N-butyl GM1 (C4)	+	+	-	yes	1348	1376
N-myristyl GM1 (C14)	+	+	-	yes	1488	1516
N-arachidyl GM1 (C20)	+	+	-	yes	1572	1600
N-cerotyl GM1 (C26)	+	+	-	yes	1656	1684

Table 1. Characterization of gangliosides and ganglioside derivatives. C18:0, 18 carbon saturated fatty acid; d18:1, sphingosine; d20:1,eicosasphingosine (20 carbons, 1 double bond).

(2) Objective #2 *Although the work is in progress, it is slow and at risk of being diffuse and poorly focused..... trafficking studies not very satisfying.....nothing on dopamine transporter* – At the reviewer's suggestion, we limited, for the results reported here, the assay of viability to the MTT assessment.

The intracellular trafficking of exogenous ganglioside derivatives is being approached in collaboration with Dr. Wayne Lencer and Dr. Anne Wolf, Childrens Hospital, Boston, MA. These investigators are utilizing a glioma cell line for their studies. They have found that, indeed, the plasma membrane uptake kinetics and cholera toxin binding of the short chain ceramide fatty acid derivative (N-butyl GM1) is significantly different than that for the parent compound. Conditions for the uptake kinetics and subcellular trafficking are being defined in the glioma cell line and will then be applied to the SH-SY5Y cells. This approach is being taken in the interest of efficient allocation of time and resources.

Further, we are aware of the role that the dopamine transporter plays in MPP+ toxicity. The transporter is present in the SH-SY5Y cells but we have no evidence that the toxin alters its gene expression.

(3) Objective #3 and Objective #4 *Considered as not yet addressed* – These objectives have not been addressed. The loss of our lysoGM1 seriously delayed synthesis of the radiolabled ganglioside derivatives needed for the BBB studies. The endothelial cell model of the BBB is now functional and the studies on BBB permeance will be initiated upon acquisition of the necessary labeled compounds. We anticipate these derivatives will be available within the next month barring unforeseen circumstances. Objective #4 will be initiated immediately upon identification of an effective cytoprotective derivative that crosses the BBB model effectively.

Report

This proposal consists of 4 specific objectives in the **Statement of Work.**

Statement of Work, Objective 1- Semisynthetic ganglioside derivatives will be synthesized from gangliosides isolated and characterized in the P.I.'s laboratory. These will include derivatives of the ceramide fatty acids, oligosaccharide functional groups, including internal esters, asialo, and reduced carboxylic acid (gangliosidol), and combinations of ceramide and oligosaccharide derivatives. Syntheses will be performed as described in Methods. Semisynthetic ganglioside derivatives will be characterized by chemical and mass spectrometric techniques.

Techniques for the synthesis of GM1 derivatives are now well established in this laboratory. In this reporting period, one serious problem arose that resulted in the destruction of the total lysoGM1 supply that had been synthesized in this project. LysoGM1 is the starting material for all of the GM1 fatty acid derivatives, both unlabeled and radiolabled. This compound is sensitive to acid hydrolysis and must be handled in a neutral or slightly alkaline condition. Chloroform purchased from a reputable source (Fisher Scientific) contained acid well beyond product specifications. This problem was not discovered for some time because the visual consequences manifested themselves identically to problems with chromatographic purification. This source of chloroform has been used in this laboratory for two decades with no previous problems. When replacement chloroform was ordered, the acidic condition still existed even though the company was aware of the problem and even though it had assured us the problem was resolved. Currently, we are negotiating with Fisher Scientific to obtain compensation for the lost time, materials, and starting material.

Statement of Work, Object 2 - Semisynthetic derivatives that specifically retain or improve the cytoprotective properties of the parent compound will be determined by testing them *in vitro* using an MPP^+ model of neurotoxicity in SH-SY5Y cells. Cytoprotection will be evaluated by use of the MTT assay, trypan blue exclusion with cell counting, and neurochemical analysis of DA, DOPAC, and HVA.

The ability of GM1 (no preincubation) to protect RA-differentiated SH-SY5Y cells from MPP^+ toxicity was determined with the MTT assay 48 hr after toxin exposure (Figure 1). The MTT assay provides an evaluation of mitochondrial status. Experiments consisted of at least 4 wells per data point and the experiment was performed in triplicate.

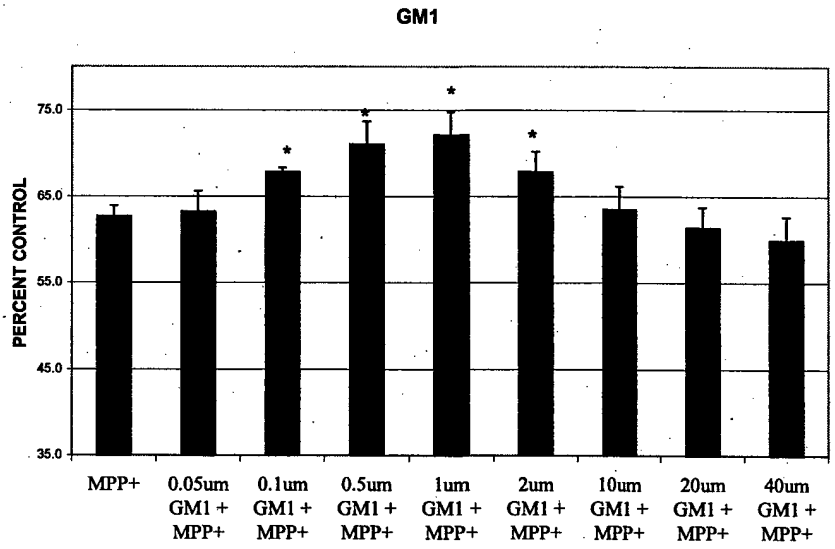

Figure 1. GM1 cytoprotection. Increasing concentrations of GM1 partially protected SH-SY5Y cells from MPP^+ (1mM) toxicity. Concentrations at or above 2uM did not provide protection.
Values represent the mean $\pm$ SEM of 4 wells
* $p<0.05$

GM1 provided partial protection against MPP^+ toxicity with maximal protection at 1 uM. Higher GM1 concentrations were not protective. The mechanism of this loss is unknown but may be due to the detergent effect of the amphipathic GM1. This phenomenon was seen with all of the derivatives tested and will earn further investigation in another project. Incubation of GM1 alone with SH-SY5Y cells under these conditions did not significantly alter cell viability compared to no GM1 controls.

The literature indicates that gangliosides may require a preincubation period prior to addition of the toxin. We tested the need for preincubation with the parent GM1. The SH-SY5Y cells were tested at 48 h after MPP+ exposure. The cells were preincubated with GM1 for 2 hr before toxin addition and

the amount of protection was compared to that with no preincubation and to the presence of toxin alone (Figure 2). Preincubation did not improve protection. Shorter (0.25 hr and 0.5 hr) and longer (4 hr and 6 hr) incubation times were also examined in a separate experiment with no improvement in cytoprotection compared to no preincubation controls. Therefore, for the MPP^+ experiments, no preincubation of GM1 or the derivatives was used.

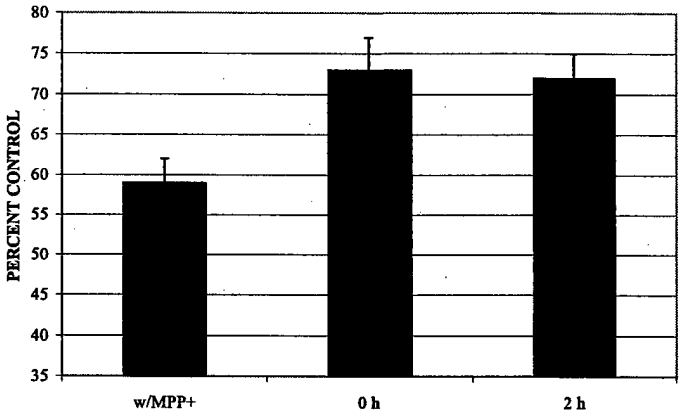

Figure 2. Preincubation of the GM1 is not required for cytoprotection.
Values are mean ± SEM
* different from MPP^+ alone, $p<0.01$, N = 6 wells

Although preincubation is not required for GM1 in this experimental paradigm, preincubation experiments to investigate optimal preincubation intervals for each new derivative to be tested are included in the experimental design.

Subsequent to establishing conditions for GM1, several GM1 derivatives have been tested *in vitro* using the MTT assay. For each derivative tested, dose response and preincubation curves were run. Cell culture plates (48-well) were utilized and each data point consisted of either 4 or 6 wells depending on the experimental design and each experiment was run in duplicate or triplicate with the

exception of the GM1 inner ester which has been run only once to this time. For brevity, comparisons are presented at the derivative concentration with maximal cytoprotection (Figure 3). All GM1 derivatives were cytoprotective with the exception of the asialo derivative, implying that the sialic acid is an important constituent for cytoprotection. LysoGM1, N-butylGM1, and N-myristylGM1 cytoprotect comparable to GM1 but their effective concentrations are significantly less than that for GM1 (lysoGM1 0.1 μM; butylGM1 0.05 uM; N-myristylGM1 0.04 uM). LysoGM1 (no ceramide fatty acid) was unexpectedly protective. This could imply that the fatty acid is not important in the cytoprotective mechanism or that the cytoprotective mechanism is

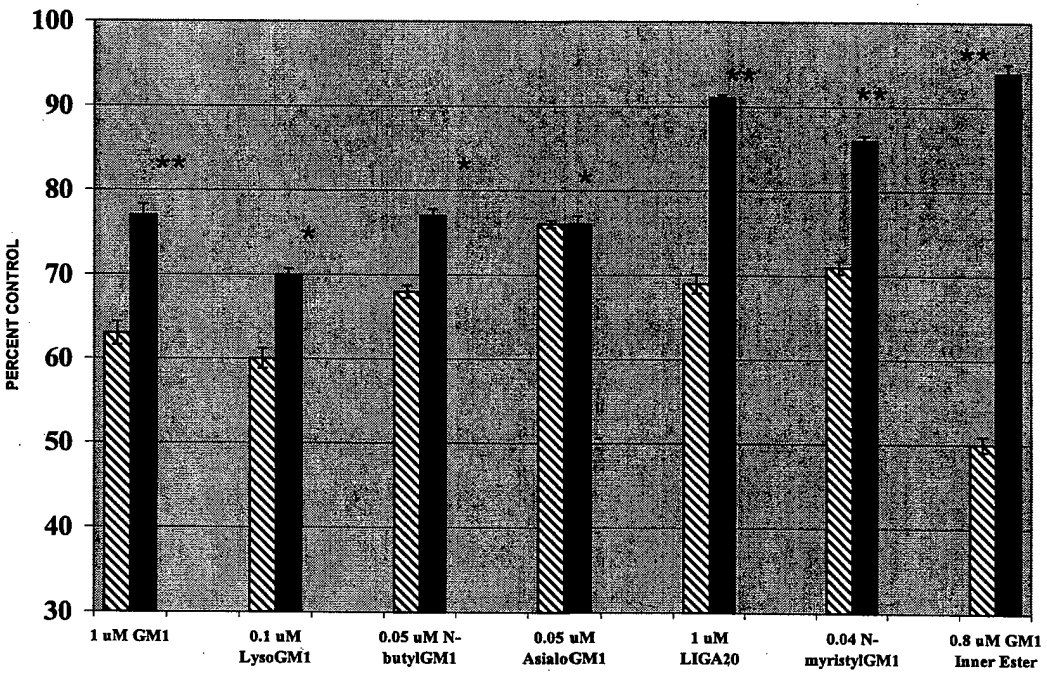

Figure 3. Comparisons of GM1 derivative cytoprotection. Values are expressed as percent control ± SEM. Stripped columns are percent control of MPP^+-treated RA-differentiated SH-SY5Y cells. Filled columns are percent control ± SEM of MPP^+-treated cells in the presence of GM1 or GM1 derivative. Differences between MPP^+ with or without GM1 or GM1 derivatives are significant except for asialoGM1.
*$p<0.05$
**p,0.01

different for lysoGM1. The likelihood of different mechanisms of action is supported by the fact that different chain length ceramide fatty acid gangliosides differentially regulate growth factor signals

(23). Also, the ceramide fatty acid is apparently important in the determination of BBB permeance. Experiments with combinations of derivatives will be tested for any additive effects of separate mechanisms. The protection afforded by the GM1 internal ester appears to be the most potent. Internal esters of the other derivatives are being prepared. Considerations for *in vivo* testing will include blood-brain barrier permeance and effective concentration rankings.

Additional experiments have been performed (in collaboration with Dr. Kelly Conn, Dr. John Wells, Dr. Richard Fine at the VA Hospital, Bedford, MA) to further establish the mechanism of MPP^+ toxicity, which will help in the delineation of mechanisms by which ganglioside derivatives are cytoprotective. To further define MPP^+ toxic mechanisms and Parkinson's disease neuronal degeneration, RT-PCR analysis was used to characterize the transcriptional response of retinoic acid-differentiated SH-SY5Y cells to MPP^+ exposure. We have shown that the protein disulfide isomerase (PDI) family member pancreatic protein disulfide isomerase (PDIp), previously considered exclusively expressed in pancreatic tissue, is uniquely upregulated among PDI family members within 24 hours following exposure of retinoic acid-differentiated SH-SY5Y cells to 1 mM MPP^+ or to 10uM of the proteasomal inhibitor lactacystin (Appendix, Item #1). Further, lactacystin treatment increases clusterin expression. Clusterin (apolipoprotein J) is a highly conserved, multifunctional vertebrate glycoprotein. Several isoforms exist and the predominant secreted isoform (sCLU) is associated with cell death and binds a variety of partly unfolded, stressed proteins including those associated with Lewy bodies in Parkinson's disease (Appendix, Item #2). The development of familial and sporadic Parkinson's disease has been associated with the ubiquitin-proteasome system dysfunction and aberrant protein degradation. These findings on altered gene expression in experimental models of Parkinson's disease provide insight into possible protective mechanisms.

In a collaborative study with Dr. Wayne Lencer and Dr. Anne wolf, we have been looking at exogenous ganglioside trafficking as suggested by a reviewer of the previous report. Initial studies have been performed in a glioma cell line. Preliminary results indicate that the shorter chain

ganglioside (N-butylGM1) associates more rapidly with the plasma membrane than does the parent GM1 but that the parent GM1 is a more effective receptor for cholera toxin subunit than the butyl derivative. These experiments are being repeated and the intracellular target for the bound cholera toxin is now under investigation. When conditions are well established, the uptake kinetics and intracellular trafficking will be examined in the SH-SY5Y cells.

Statement of Work, Objective 3, Effective cytoprotective semisynthetic ganglioside derivatives that effectively cross a brain capillary endothelial cell model of the blood-brain barrier (BBB) will be determined. Model BBB transcytosis will be assessed by liquid scintillation counting of radiolabeled derivatives in aliquots taken from the lower wells of Transwell cell culture plates. These studies have been delayed because of the lysoGM1 loss described above. Radiolabeled derivatives are now being synthesized using galactose oxidase and [^{3}H]-sodiumborohydride and the experiments will soon begin.

Statement of Work, Objective 4, Semisynthetic ganglioside derivatives that effectively protect neurons *in vivo* using chronic and acute MPTP administration models of neurotoxin insult will be determined by testing, in mice, those derivatives that both cytoprotect the SH-SY5Y cells from MPP^+ toxicity *in vitro* AND that effectively cross the *in vitro* BBB model. The chronic and acute models represent apoptotic and necrotic cell death mechanisms, respectively. Neuroprotection will be evaluated by neurochemical analysis of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), and by neuronal counts of Nissl substance, tyrosine hydroxylase (TH) and dopamine transporter (DAT) positive neurons of the substantia nigra pars compacta. Work on this objective is also delayed but will begin upon completion of the BBB studies.

KEY RESEARCH ACCOMPLISHMENTS

- Increasing concentrations of GM1 partially protects SH-SY5Y cells from MPP^+ (1mM) toxicity. Concentrations at or above 2uM appeared to make the cells more vulnerable to MPP^+ toxicity.
- Preincubation of the GM1 is not required for cytoprotection
- Ganglioside derivatives provide cytoprotection comparable to the parent GM1 but at lower concentrations
- GM1 protects against low potassium induced apoptotic cell death
- MPP^+ or lactacystin upregulate the expression of PDIp
- Lactacystin increases clusterin expression

REPORTABLE OUTCOMES

Manuscripts

Conn,K., Ullman MD, Larned MJ, Eisenhauer PB, Fine RE, Wells JM: cDNA Microarray Analysis of Changes in Gene Expression Associated with MPP^+ Toxicity in SH-SY5Y Cells, *Neurochem. Res.* Neurochemical Research (2003) 28: 1873-1881

Conn KJ, Gao W, McKee A, Lan MS, Ullman MD, Eisenhauer PB, Fine RE and Wells JM. Identification of the Protein Disulfide Isomerase Family Member PDIp in Experimental Parkinson's Disease and Lewy Body Pathology. Brain Research. In press.

Carreras I, Garrett-Young R, Ullman MD, Eisenhauer PB, Fine RE, Wells JM, Conn KJ. Upregulation of clusterin/apolipoprotein J in Lactacystin-treated SH-SY5Y Cells. J. Neurosci. Res. Submitted.

Abstracts

Conn KJ, Garrett-Young R, Ullman MD, Gao WW, Eisenhauer PB, Fine RE, Wells JM. Upregulation of Clusterin/apolipoprotein J in Lactacystin-treated SH-SY5Y Cells. 9th International Conference on Alzheimer's Disease and Related Disorders, July 17-22, 2004

Conn KJ, Carreras I, Ullman MD, Eisenhauer PB, Fine RE, Wells JM. Upregulation of Growth/Differentiation Factor-15 (GDF-15)/Macrophage Inflammatory Peptide-1 (MIC-1) by MPP^+ and Lactacystin in differentiated SH-SY5Y Cells. Society for Neuroscience, 34th Meeting, San Diego, CA 2004

Presentations

Ullman MD. Neuroprotective Ganglisode Derivatives: An Overview. Department of Health and Environmental Sciences, University of Massachusetts Lowell, Lowell, MA. April 21, 2004

CONCLUSIONS

Our experience in synthesizing and developing HPLC procedures to purify semisynthetic ganglioside derivatives provides the opportunity to find improved cytoprotective ganglioside derivatives, to define required functional groups for cytoprotection, and to initiate studies to determine cytoprotective mechanisms. Regrettably, the studies have been impeded by the unfortunate and unavoidable loss of

the lysoGM1 supply. Still, progress has been made in the *in vitro* studies. GM1, to approximately 2uM) partially protected SH-SY5Y neuroblastoma cells from MPP^+ toxicity. GM1 concentrations above 2uM appeared to make the cells more vulnerable to MPP^+ toxicity and this phenomenon warrants further investigation in another study. Preincubation of the GM1 was not required. Ganglioside derivatives provide comparable cytoprotection but at lower concentrations than that for the parent compound. These compounds will be tested in the endothelial cell model of the BBB.

Experiments have been performed to further establish the mechanism of MPP^+ toxicity, which will help in the delineation of mechanisms by which ganglioside derivatives are cytoprotective. Experimental modeling of Parkinson's disease neurodegeneration using MPTP and MPP^+ identified changes in gene expression of different endoplasmic reticulum stress proteins associated with MPTP- and Parkinson's disease-related neurodegeneration. The protein disulfide isomerase (PDI) family member pancreatic protein disulfide isomerase (PDIp) was found to be uniquely upregulated among PDI family members following exposure of retinoic acid-differentiated SH-SY5Y cells to MPP^+ or to the proteasomal inhibitor lactacystin. Further, lactacystin treatment increased clusterin expression. The development of familial and sporadic Parkinson's disease has been associated with the ubiquitin-proteasome system dysfunction and aberrant protein degradation. These findings on altered gene expression in experimental models of Parkinson's disease emphasize the importance of understanding the mechanisms of MPP^+ toxicity in attempts to further define mechanisms of ganglioside neuroprotection because by better understanding neurotoxic and neuroprotective mechanisms, specific steps in the cell death process can be targeted.

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APPENDICES

1. Journal article

Conn KJ, Gao W, McKee A, Lan MS, **Ullman MD**, Eisenhauer PB, Fine RE, Wells JM: Identification of the protein disulfide isomerase family member PDIp in experimental and idiopathic Parkinson's disease. *Brain Research*. In press.

2. Journal Article

Carreras I, Garrett-Young R, **Ullman MD**, Eisenhauer PB, Fine RE, Wells JM, Conn KJ. Upregulation of Clusterin/Apolipoprotein J in Lactacystin-treated SH-SY5Y Cells. Submitted to *Journal of Neuroscience Research*

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Identification of the protein disulfide isomerase family member PDIp in experimental Parkinson's disease and Lewy body pathology

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Abstract

Parkinson's disease (PD) is a slowly progressing neurodegenerative disorder with no clear etiology. Pathological hallmarks of the disease include the loss of dopaminergic neurons from the substantia nigra (SN) and the presence of Lewy bodies (LBs) (α-synuclein and ubiquitin-positive, eosinophilic, cytoplasmic inclusions) in many of the surviving neurons. Experimental modeling of PD neurodegeneration using the neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenyl-pyridinium (MPP^+) has identified changes in gene expression of different endoplasmic reticulum (ER) stress proteins associated with MPTP- and PD-related neurodegeneration. We show that the protein disulfide isomerase (PDI) family member pancreatic protein disulfide isomerase (PDIp), previously considered exclusively expressed in pancreatic tissue, is uniquely upregulated among PDI family members within 24 h following exposure of retinoic acid (RA)-differentiated SH-SY5Y human neuroblastoma cells to either 1 mM MPP^+ or 10 μM of the highly specific proteasome inhibitor lactacystin. RT-PCR confirms PDIp expression in brain of post-mortem human PD subjects and immunohistochemical studies demonstrate PDIp immunoreactivity in LBs. Collectively, these findings suggest that increased PDIp expression in dopaminergic (DA) neurons might contribute to LB formation and neurodegeneration, and that this increased PDIp expression may be the result of proteasome impairment.

Theme: Disorders of the nervous system
Topic: Degenerative disease: Parkinson's

Keywords: 1-Methyl-4-phenyl-pyridinium; Parkinson's disease; Lewy body; Dementia with Lewy bodies; Pancreatic protein disulfide isomerase

1. Introduction

Parkinson's disease (PD) afflicts about 1% of people over age 65 and 4–5% of people over age 85, making it the most common movement disorder and the second most common neurodegenerative disorder in humans [21]. Because of its prevalence, PD is a major societal health problem. Presently, there is no cure, diagnostic marker, or clear etiology for the disease. The pathological hallmarks of PD are the degeneration of dopamine (DA)-containing neurons in the substantia nigra (SN) and the presence of Lewy bodies (LBs) in many of the surviving DA neurons [11].

LBs are 5–25 μm hyaline intraneuronal inclusions classically consisting of three layers of varying eosinophilia [13]. LBs are found in neurons in several neurodegenerative

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disorders such as PD, Dementia with Lewy bodies (DLB), and Alzheimer's disease (AD) [11,4]. LBs contain a variety of proteins including phosphorylated neurofilament, ubiquitin, and α-synuclein [11,29,33].

The majority of PD cases are sporadic; however, the identification of a number of genes associated with rare familial forms of PD has provided important insights into the underlying mechanisms of the disease. These genes, encoding α-synuclein, parkin, UCH-L1, DJ-1, and PINK1 have implicated protein misfolding, oxidative stress, and impairment of the ubiquitin-proteasome system (UPS), in the pathogenesis of the disease [14,25,4,5,35]. The ubiquitin-proteasome system (UPS) is the primary biochemical pathway responsible for the degradation of normal and abnormal intracellular proteins [15,16]. Proteolysis by the proteasome is specifically inhibited by the Streptomyces metabolite lactacystin [9]. UPS failure leads to protein accumulation and cell death [32,36].

The accumulation of misfolded/unfolded proteins within cells triggers an endoplasmic reticulum (ER) stress response [18]. Cellular changes associated with the ER stress response include the unfolded-protein response (UPR). The UPR is characterized by the induction of gene expression of ER-localized protein-folding catalysts and protein chaperones and also by the inhibition of protein translation by phosphorylation of the eukaryotic initiation factor-2α (eIF-2α). Under conditions of severe ER stress, the apoptotic cell death pathway is activated [18].

We [3] and others [17,31] have recently demonstrated that the UPR gene GADD153 is upregulated in the 1-methyl-4-phenyl-pyridinium (MPP^+) neurotoxin model [30] of PD neurodegeneration. As PDI family members are also upregulated by the UPR [18], we investigated the expression of the PDI family of proteins in toxic models of PD neurodegeneration and in human brain tissue from PD and DLB subjects in the current study. The PDI family of proteins consists of structurally related enzymes that catalyze disulfide bond formation, reduction, or isomerization of newly synthesized proteins [10,12,34]. The PDI family also acts as part of a quality-control system to ensure the correct folding of proteins, a feature essential to cell viability.

The results of this study demonstrate that PDIp is uniquely upregulated among family members by either MPP^+ or lactacystin. RT-PCR confirms PDIp expression in human brain and immunohistochemical studies demonstrate that PDIp immunoreactivity in LBs co-localizes with α-synuclein.

2. Materials and methods

2.1. Materials

MPP^+ iodide, lactacystin, canavanine, Dulbecco's Modified Eagle's Medium (DMEM), retinoic acid (RA), Tri reagent, (Sigma, St. Louis, MO), H_2O_2 (Fisher Scientific, Rochester, NY), and fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) were purchased from commercial sources.

2.2. Cell culture and toxin treatment

The undifferentiated human neuroblastoma cell line SH-SY5Y (ATCC CRL-2266) was cultured at 37 °C in a 95% air, 5% CO_2 humidified incubator and maintained in DMEM (high glucose) supplemented with 10% FBS. Undifferentiated cells were routinely subcultured when confluent and the culture medium was changed twice a week. For toxin experiments, cells were plated into 48-well plates (10,000 cells/well) and/or 100 mm^2 dishes (0.8×10^6 cells/dish) in differentiation media (10 ml DMEM plus 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin and 10 μM RA). Cells were cultured for 3 days then the media were changed to fresh differentiation media. The cells were cultured another 3 days and freshly prepared toxin or vehicle was added in fresh differentiation media for various lengths of time. All appropriate safety precautions were used in handling toxin solutions.

2.3. RNA extraction and RT-PCR

Total RNA was isolated from 100 mm^2 dishes in 1 ml Tri Reagent (Sigma). Approximately 50 μg of RNA was treated with 1 unit of DNAse-I using the MessageClean kit (GenHunter, Nashville, TN). DNAse-treated RNA was subjected to RT-PCR analyses, as described below, using primers to glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; G3PDH) to ensure that each RNA preparation was free of DNA. DNAse-treated RNA (5 μg) was reverse transcribed using oligo(dT) primers provided in the Superscript kit (Clontech, Palo Alto, CA). Three different primer sets were used for the amplification of PDIp (5′CGCCTGGTCACGGAGTTC 3′and 5′CCACAGGCGCATACTTCTTAGT 3′), (5′ CTTTGGACTCAAGGCTGAGG 3′ and 5′ GCTCCTGTCCAGCAGTGATG 3′), and (5′ AGCCTCCAGAGGAGGAAATC 3′ and 5′ AGGTCCTGGAAGAAGCCAAT 3′). The following primers were purchased (Gibco BRL) and used for the detection of the PDI family members: PDI (5′ AGACTCACATCCTGCTGTTCTTGCC 3′ and 5′ TCGGGCTTGTACTTGGTCATCTCC 3′), PDIR (5′ GAAGAGCAGCAGACAAGCCTGTTGC 3′ and 5′ AAATCCCAATTCTGTGCGGTCGCTG 3′), P5 (5′ CAGTCTGAACTTGAGACCGCGTTGG 3′and 5′GTCATCAAGCTCCACATCACTGAGG 3′), ERp29 (5′ TCCCAAAAGCAAGTTCGTCT 3′and 5′ GCTCCATGTTCAGCTTGTCA 3′), ERp44 (5′TCTTGATCGCAGCAAAAGAA 3′and 5′ACCATATCCGGAGCAGAATG 3′), ERp57 (5′ TTATTTGTGTTGGGGGAAATG 3′ and 5′ AGGGGTCTCATTTATTGTCACTG 3′), ERp72 (5′ GCATCGTTTGACACCTTGCGGTGG 3′ and 5′ CAATACCAGGATGCCGCTAACAACC 3′). Conditions for G3PDH amplification were identical to those previously

described [3]. For each primer set the reaction cycle consisted of the following steps: 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min. The number of PCR cycles needed for each primer set was optimized to give PCR products in the linear range of detection for the ChemiImaging system described below. Reactions for G3PDH, PDI, P5, PDIR, ERp29, ERp72 were carried out for 30 cycles while reactions for PDIp, ERp44, and ERp57 were carried out for 35 cycles. Following resolution by electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide, PCR products were visualized and quantified using the 4400 ChemiImager low light imaging system (Alpha-Innotech, San Leandro, CA). PDIp, PDI, PDIR, ERp29, ERp44, ERp57, ERp72 expression was expressed as a ratio to the value of G3PDH product obtained from parallel reactions. RT-PCR confirmation of PDIp expression in human brain used RNA isolated from frozen, post-mortem, superior frontal cortex from a subject with pathologically confirmed DLB (Harvard Brain Tissue Resource Center). RT-PCR was also performed using cDNA generated from frozen, post-mortem, whole brain lysates from a control subject with non-neurological diseases (Ambion, Austin, TX).

2.4. Western blots

Cells were harvested from 100 mm^2 dishes in 300 μl of phosphate buffered saline (PBS) using a plastic cell lifter. Harvested cells were pelleted by centrifugation (12,000×*g* at 4 °C), resuspended in 100 μl lysis buffer (20 mM Tris pH 7.4, 140 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1× protease inhibitor cocktail (Roche, Mannheim, Germany) and frozen to −70 °C. Thawed cell suspensions were clarified by centrifugation (12,000×*g* at 4 °C). The protein concentrations of the resultant supernatants were determined using the BCA kit (Pierce, Rockford, IL). Fifty μg of protein were resolved on a 10–20% Tris–HCl gel and transferred to nitrocellulose (Bio Rad, Hercules, CA). After transfer, gels were stained with Bio-Safe Coomassie reagent (Bio Rad) to monitor equal protein loading of lysates from the different experimental conditions. Membranes were blocked in 5% non-fat dried milk in TBST buffer (20 mM Tris, 500 mM NaCl pH 7.5 with 0.05% Tween-20) for 60 min at room temperature. Membranes were then washed for 10 min three times in TBST. Membranes were incubated with 1:200 dilutions of polyclonal rabbit anti-PDIp antisera [7] or polyclonal rabbit anti-PDI (Stressgen Biotechnologies, Victoria, BC, Canada) diluted in TBST overnight at room temperature. Membranes were washed as before and incubated with a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (Chemicon International, Temecula, CA) diluted in TBST for 2 h at room temperature. Membranes were washed as before and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce). Membranes were visualized with the 4400 ChemiImager low light imaging system (Alpha-Innotech). Exposure times were optimized to collect images of protein products within the linear range of detection for the Chemiimaging system.

2.5. Immunohistochemistry

Human brain tissue from subjects with pathologically confirmed PD (five cases; ages 63–86 years; mean age, 78.6 years), DLB (eight cases; ages 74–87 years; mean age, 80.1 years), AD (six cases; ages 75–83 years; mean age, 78 years), and control subjects with non-neurological diseases without any pathological changes associated with PD, DLB or AD (four cases; ages 73–86 years; mean age, 78.2 years) were obtained from the Boston University Brain Tissue Resource Center and used for immunocytochemistry. Sections from fresh-frozen and paraffin embedded tissues were incubated with either blocking serum (same species as secondary antibody) for 20 min or Super Block solution (ScyTek Laboratories, UT) for 5 min. Sections were incubated with either polyclonal rabbit anti-PDIp antisera [7] and/or mouse monoclonal anti-α-synuclein antibody (Zymed Laboratories; South San Francisco, CA) overnight at 4 °C. Sections were washed in PBS plus 10% BSA prior to incubation with biotinylated secondary antibodies (Vector, CA), Alexa Flour 488 goat anti-rabbit IgG (Molecular Probes; Eugene, OR) (PDIp) or Alexa Flour 546 anti-mouse IgG (Molecular Probes; Eugene, OR) at room temperature for 1 h. The sections were washed as before and staining was performed with an avidin–biotin complex (RTU ABC elite kit, Vector, CA) and AEC system (ScyTek Laboratories, UT) following manufacturers' instructions or detected by fluorescence microscopy (Nikon Eclipse E800 with RT Slider). Some sections were also counterstained with hematoxylin or 4′,6-diamidino-2-phenylindole*2HCl (DAPI). The specificity of anti-PDIp serum was also tested by preabsorption assay (data not shown) using anti-PDIp serum (1:500 in PBS plus 10% BSA) preabsorbed with 20 μg/ml human (6xHis)-PDIp peptide, room temperature for 1 h before applied as primary antibody.

2.6. Analysis of cell viability

Cells were plated into 48-well plates (10,000 cells/well) and differentiated as described above and freshly prepared toxins were added for 24 h. The conversion of soluble 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to insoluble formazan was assayed using the CellTiter Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) as described previously [23].

2.7. Statistical analysis

Values represent the means±SEM. Differences among means were analyzed by Student's *t*-test or one-way ANOVA with time or treatment as the independent factor. When ANOVA showed significant differences, post-hoc tests were performed.

3. Results

3.1. MPP^+ exposure and mRNA expression

Steady-state mRNA levels of each PDI family member were measured using RT-PCR with primers and RNA isolated 24, 48 and 72 h after exposure of differentiated SH-SY5Y cells to 1 mM MPP^+ or control conditions from their respective no toxin control. The expression of PDIp is increased at all timepoints but that of the family members PDI, PDIR, P5, ERp29, ERp44, ERp57 or ERp72 is not (Fig. 1A). Quantification of steady-state PDIp mRNA levels from four independent experiments demonstrated that the increase in PDIp in MPP^+-treated cultures after 24 h is 240.8±43.1% (mean±SEM, one sample *t*-test, $p<0.05$). One-way ANOVA on the individual gene comparisons showed that PDIp was uniquely upregulated. PDIp expression did not differ significantly between 24, 48, and 72 h (Fig. 1B).

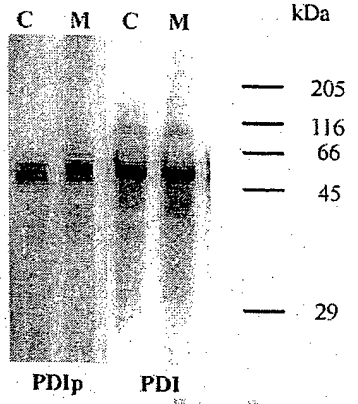

Fig. 2. Western blot analysis of PDIp and PDI expression in differentiated SH-SY5Y cells-Western blot analyses were performed using cell extracts isolated 24 h after MPP^+ exposure. Shown are results from a typical experiment in which lysates from control (C) and MPP^+-treated cells (M) were electrophoretically separated, transferred to nitrocellulose and probed with antibodies to PDIp (left panel) and PDI (right panel). Molecular weight standards in kilodaltons (kDa) are shown to the right of the blots.

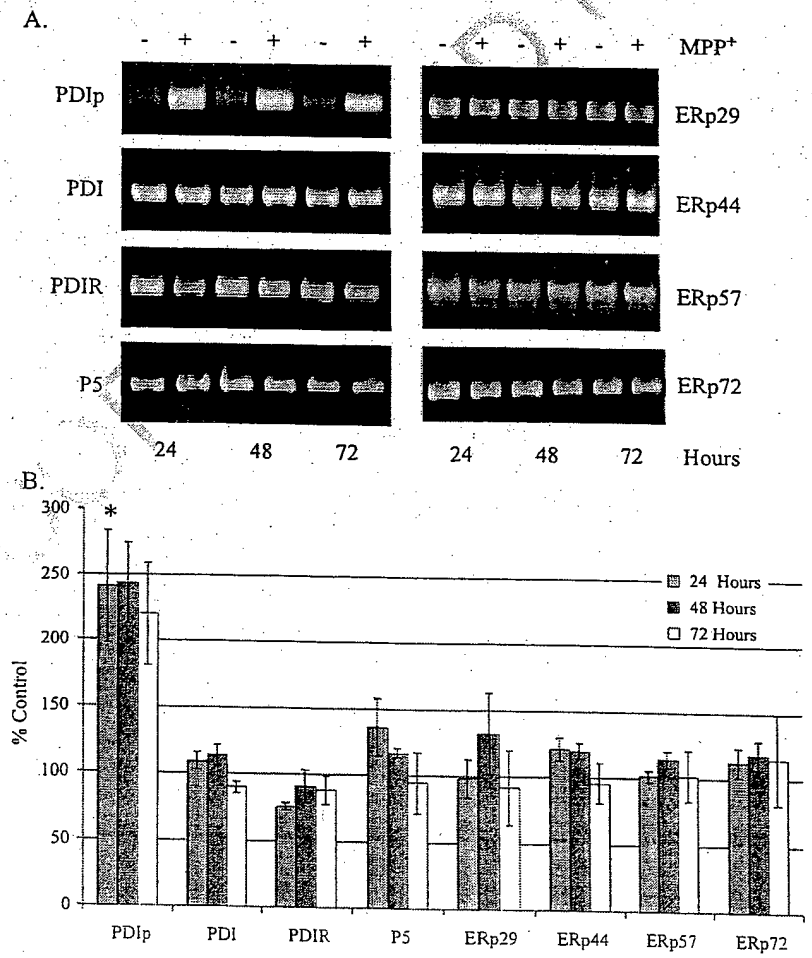

Fig. 1. RT-PCR analysis of PDIp and other PDI family member expression in differentiated SH-SY5Y cells. (A) Electrophoretic separation of PCR products from a typical RT-PCR experiment is shown using primers to PDIp, PDI, PDIR, P5, ERp29, ERp44, ERp57, ERp72 and RNA isolated 24, 48, and 72 h after exposure to 1 mM MPP^+ (+) or no-toxin control conditions (−). (B) Quantification of PDIp, PDI, PDIR, P5, ERp29, ERp44, ERp57, ERp72 steady-state mRNA was performed as described in the text. Data are graphed as % control±SEM where control represents those cells not treated with MPP^+. Values represent the mean of four independent experiments $*p<0.05$.

3.2. Western blot analysis

Western blot analysis was performed to determine the influence of MPP^+ on steady-state PDIp protein levels using protein-specific antibodies to PDIp [26] on cell lysates isolated 24 h after exposure of cells to 1 mM MPP^+ or control conditions. In parallel, steady-state PDI protein levels were also determined. Because 24 h of MPP^+ (1 mM) exposure did not alter PDI steady-state mRNA levels (Fig. 1), we did not expect steady-state PDI protein levels to change under these same experimental conditions. Both PDIp and PDI migrate with an apparent molecular weight of ~55 kDa [10]. PDIp but not PDI expression was increased in MPP^+-treated cells compared to control as shown by Western blot (Fig. 2). Coomassie staining of the SDS gels (data not shown) was done to confirm that equal amounts of protein were loaded on the gels. Quantification of steady-state PDIp and PDI protein levels from five independent experiments demonstrated that steady-state PDIp protein levels, normalized to steady-state PDI protein levels, in MPP^+-treated cultures as compared to control was increased to 129±8.82% of control (mean±SEM, two-tailed one sample t-test $p<0.05$). Comparison of PDIp steady-state protein levels among lysates isolated 4, 12, 24 and 72 h following MPP^+ exposure (1 mM) showed that differences in steady-state PDIp protein levels as compared to control were maximal at the 24 h timepoint (data not shown).

Previous studies identified PDIp as being exclusively expressed in the pancreas [6]. To confirm that the product in our cell culture model was indeed PDIp and to determine if PDIp was also expressed in human brain tissue, RT-PCR was performed using three unique primers sets to PDIp and cDNA from three sources: (1) MPP^+-treated differentiated SH-SY5Y cells; (2) human whole-brain lysates (Ambion);

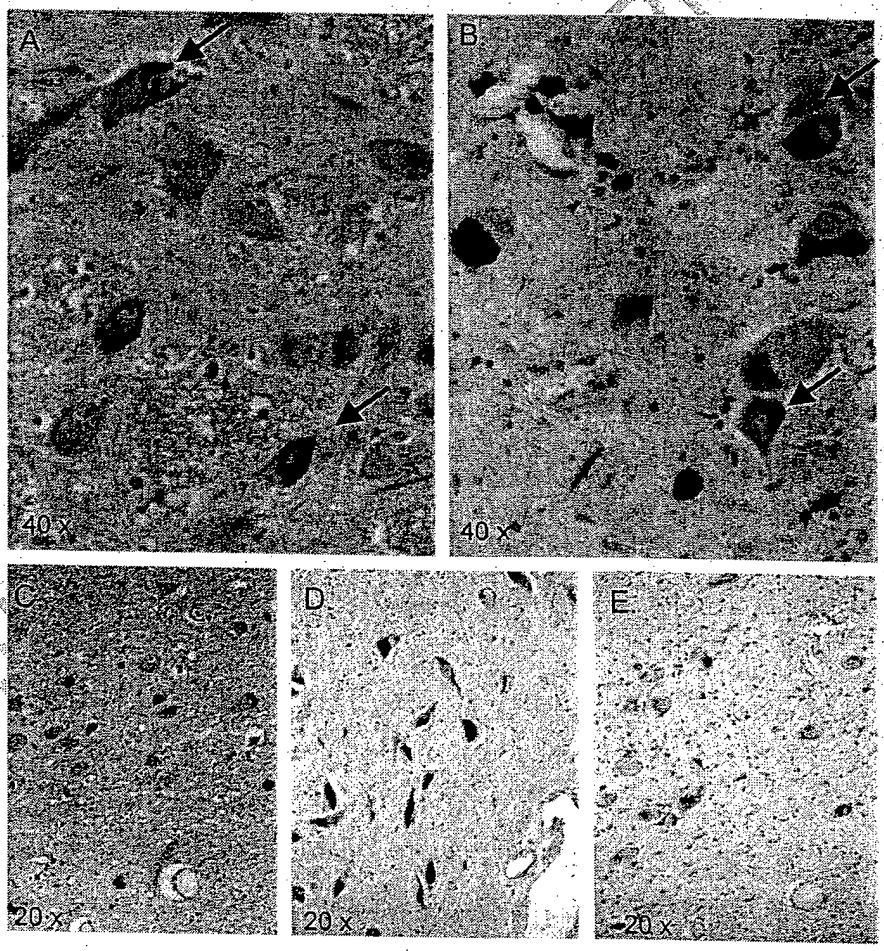

Fig. 3. PDIp immunohistochemistry in human DLB and control substantia nigra. Some 10-μm sections of DLB and control (non-diseased) brain tissues were incubated with and without antibodies to PDIp and α-synuclein. Immunoreactivity was detected using a vector ABC staining kit with AEC chromogen. The sections were also counterstained by hematoxylin. (A) Substantia nigra from DLB brain immunostained with antibodies to PDIp (40× original magnification) with typical brainstem LBs indicated by arrows. The rounded area of neuromelanin clearing indicates the position of the Lewy bodies in several neurons of the substantia nigra. Smaller dot-like inclusions in the area of the melanin clearing are immunoreactive for PDIp suggesting that PDIp stains only a portion of the LB structure. B. Substantia nigra from DLB brain immunostained with antibodies to α-synuclein (40×) with typical brainstem LBs indicated by arrows. (C) Substantia nigra from DLB brain immunostained with antibodies to PDIp (20×). (D) Substantia nigra from control (non-diseased) brain immunostained with antibodies to PDIp (20×). (E) Substantia nigra from DLB brain immunostained with secondary antibody only (20×).

and (3) post-mortem superior frontal cortex from a patient with DLB. All three sources of cDNA produced PCR products of the expected sizes with all three unique primer sets (data not shown). Sequencing of the PCR products produced from amplification of RNA isolated from DLB brain tissue and from MPP^+-treated differentiated SH-SY5Y cells showed 100% sequence identity to PDIp within the sequences tested (bases 84–524 and 1062–1544; gi5803118).

3.3. Immunohistochemical analysis

To determine the distribution of PDIp in human brain, immunohistochemistry was performed. Positive PDIp immunostaining localized to LBs was found in some nigral neurons of all PD and DLB subjects examined. PDIp stains discrete intraneuronal inclusions that are irregularly shaped, and are not always rounded, which most likely represents staining of only a portion of the Lewy body (Fig. 3A and C). In addition, PDIp stains irregular threadlike neuritis in the surrounding neuropil that conform to the appearance of Lewy neurites. The number of PDIp positive LBs varied from case to case. Positive PDIp immunostaining localized to LBs was also found in the amygdala, entohrinal cortex, hippocampus, and medulla of all DLB subjects examined (data not shown). There was no PDIp immunostaining in the SN of control subjects (Fig. 3D), or in the SN of DLB subjects in sections incubated with secondary antibody only (Fig. 3E). In addition, there was no PDIp immunostaining in senile plaques or neurofibrillary tangles in the brains of AD subjects (data not shown).

To estimate the subpopulation of PDIp immunoreactivity aggregates in classical Lewy bodies as defined by α-synuclein staining, 10 continuous, non-overlapping 20× fields from individually stained, serial 10 μM sections of four Parkinson's disease patients were counted. Approximately 57% of α-synuclein immunostained LBs are also immunopositive for PDIp.

To determine if PDIp and α-synuclein immunoreactivities were co-localized in LBs, a double labeling experiment was performed. Sections of nigral tissue from subjects with pathologically confirmed DLB were incubated with antibodies to α-synuclein and PDIp bound to unique fluorescent secondary antibodies. Positive PDIp (green) and α-synuclein (red) immunostaining was identified in some LBs and the overlay of the two fluorescent signals (yellow) demonstrates that in those LBs where they are both present, α-synuclein and PDIp immunoreactivity co-localize (Fig. 4). The counterstain of nuclei with DAPI (blue) is also shown.

3.4. Lactacystin exposure and PDIp expression

Because LB formation in PD and DLB is associated with impaired proteasome function [14,26], we tested whether the highly specific proteasome inhibitor, lactacystin, increased PDIp expression in differentiated SH-SY5Y cells.

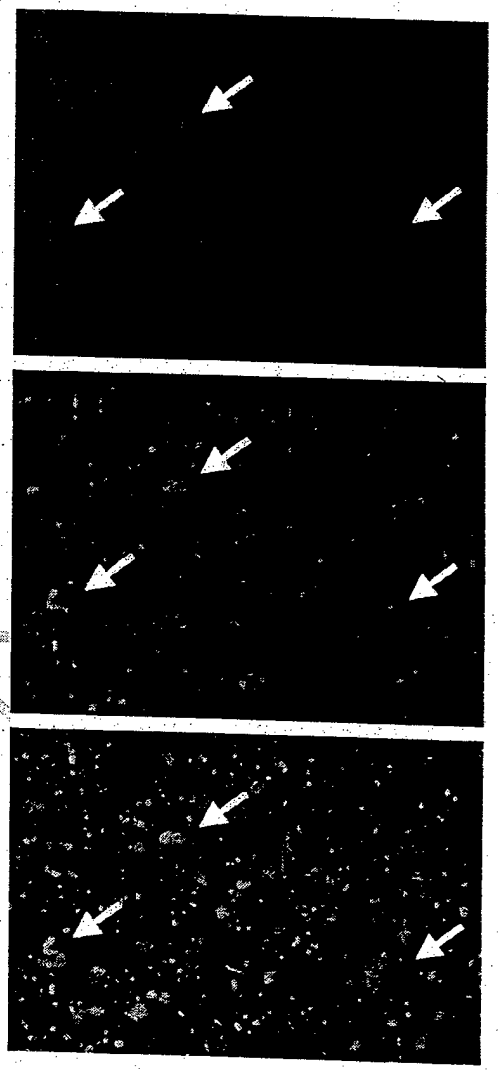

Fig. 4. Co-localization of α-synuclein and PDIp expression in human DLB, substantia nigra. A double labeling experiment was performed using DLB, SN tissue immunostained with antibodies to α-synuclein (red) and PDIp (green) each bound to unique fluorescent secondary antibodies. The red staining shows α-synuclein immunoreactivity, the green staining shows PDIp staining immunoreactivity, the yellow staining shows areas where α-synuclein and PDIp immunoreactivity overlay. The counterstain of nuclei with DAPI (blue) is also shown. Typical brainstem LBs are indicated by arrows (40× magnification).

Cells were treated with 10 μM lactacystin or no-toxin control conditions for 24 h and RNA was isolated for RT-PCR analysis (Fig. 5). Quantification of steady-state PDIp mRNA levels from three independent experiments demonstrate that lactacystin increases PDIp expression by 148.7±1.9% compared to the control (mean±SEM, one sample t-test, $p<0.002$, $n=3$). PDIp expression did not increase in differentiated SH-SY5Y cells following exposure for 24 h to the oxidant H_2O_2 (600 μM) or to the inducer of misfolded proteins canavanine (10 mM) even though both agents display similar toxicity with lactacystin (data not shown).

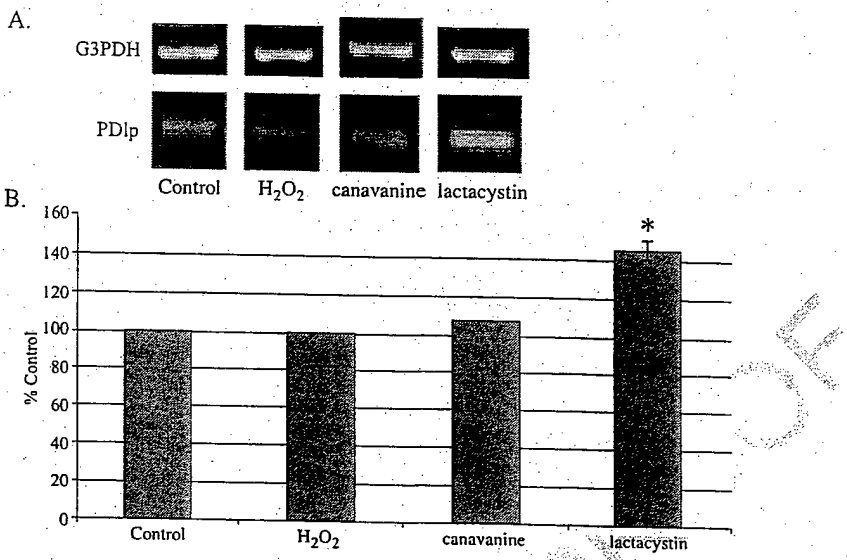

Fig. 5. Expression of PDIp in differentiated SH-SY5Y cells following exposure to H_2O_2, canavanine, and lactacystin. (A) Electrophoretic separation of PCR products from a typical RT-PCR experiment is shown using primers to G3PDH and PDIp and RNA isolated 24 h after exposure to control conditions (no toxin), H_2O_2 (600 μM), canavanine (10 mM) and lactacystin (10 μM). (B) Quantification of PDIp steady-state mRNA was performed as described in the text. Data are graphed as % control±SEM where control represents those cells not treated with MPP^+. Values for lactacystin represent the mean of three independent experiments $^*p<0.002$. Values for H_2O_2 and canavanine represent the mean of two independent experiments.

Western blot analysis was performed to determine the influence of lactacystin on steady-state PDIp and PDI protein levels using cell lysates isolated 24 h after exposure of cells to 10 μM lactacystin or control conditions. PDIp but not PDI steady-state protein levels increased following lactacystin exposure (data not shown). Quantification of steady-state PDIp and PDI protein levels from four independent experiments demonstrated that steady-state PDIp protein levels, normalized to steady-state PDI protein levels, in lactacystin-treated cultures as compared to control was increased to 130.3±3.12% of control (mean±SEM, two-tailed one sample t-test $p<0.05$).

4. Discussion

This study shows that the PDI family member PDIp is uniquely upregulated among family members within 24 h following exposure of differentiated SH-SY5Y cells to either MPP^+ or lactacystin. In contrast, neither H_2O_2 nor canavanine increased PDIp expression despite decreasing cell viability to a comparable degree. Together, this suggests that PDIp upregulation may be a specific signal for proteasomal impairment. This hypothesis is supported by our recent unpublished observation that exposure of SH-SY5Y cells to the proteasome inhibitor, MG115 [23] also increases PDIp steady-state mRNA (~140% control after 24 h exposure to 30 μM). Cell death following canavanine, we speculate, is likely due to the generation of several defective polypeptides (not just those associated with the proteasome) because canavanine (the beta-oxa-analog of L-arginine and a substrate for arginyl tRNA synthetase) is incorporated into all nascent proteins in place of L-arginine [1].

Western blot analysis of total cell lysates confirmed that PDIp steady-state protein levels are up-regulated in response to both MPP^+ and lactacystin; though the magnitude of steady-state protein upregulation was less than that of steady-state mRNA. The difference between the magnitude of induction between steady-state protein and mRNA levels has also been found with other ER stress genes [3] and may be related to the observation that induction of ER stress genes is accompanied by phosphorylation of the eukaryotic initiation factor-2α (eIF-2α), which results in an overall decrease in protein translation [18]. It remains to be seen whether the ~30% increase in steady-state PDIp protein levels following toxin exposure results in a proportional increase in PDIp activity.

It is unclear why MPP^+ and lactacystin increase PDIp expression but not the expression of other PDI family members. One possibility is that the different PDI related proteins fulfill similar functions but act on specific substrates. PDIp is structurally and functionally related to PDI. PDI and PDIp share the same domain organization (45% sequence identity) but PDIp lacks an enriched acidic amino acid stretch at the carboxyl terminus that functions as a peptide-binding domain [28]. In addition, the PDIp cDNA contains three potential N-linked glycosylation sites not found in typical mammalian PDIs. Both PDI and PDIp have been shown to specifically interact with radiolabeled model peptides and misfolded proteins in vitro; however, PDIp but not PDI has high affinity for peptides with hydroxyaryl groups [19]. Characterizing protein–protein interactions will provide insight into the possible protein substrates of brain PDIp.

Alternatively, the members of the PDI family might be differentially regulated. For example, some PDI-related proteins are upregulated by the UPR [22,24]. Future identification of the responsive promoter element(s) of a PDIp gene may provide further insights into the regulatory molecules that signal the initial events in dopaminergic degeneration. In addition, if upregulation of PDIp is found to be exclusively located with in the dopaminergic cells in the SN, characterization of the PDIp promoter elements may provide the basis for expressing protective genes in dopaminergic cells affected in PD.

In addition to being expressed in SH-SY5Y cells, PDIp steady-state mRNA was detected in human brain by RT-PCR. The sensitivity of RT-PCR to detect low levels of mRNA transcripts may account, in part, for our discovery that PDIp is expressed in the brain. This result contrasts with Northern blot experiments that suggested PDIp expression was restricted to pancreatic tissue [6]. We have used two methods to help clarify whether PDIp immunoreactivity in the brain represents neuronal expression. First, we have used laser capture microdisection to isolate RNA from Lewy body containing neurons of the substantia nigra and used this RNA to detect PDIp expression by RT-PCR (unpublished results). Secondly, we have cultured mouse cortical neurons and have shown that MPP^+ increases the expression of murine PDIp in these primary neuronal cultures (unpublished results).

Positive PDIp immunostaining localized to LBs was found in some nigral neurons of all PD and DLB subjects examined. This restricted distribution of PDIp immunoreactivity may be the result of increased PDIp expression in response to ongoing exposure to toxic factor(s) in those neurons and/or the sequestration of PDIp to the LB over time. PDIp immunoreactivity is detected in both brainstem and cortical LBs and co-localizes with α-synuclein immunoreactivity. The co-localizaiton of α-synuclein and PDIp suggests that these molecules may have close physical interactions. Although it is not clear why only some LBs are immunopositive for PDIp, it may represent the existence of different stages of LBs development. Sequestration of PDIp within the LB could lead to a loss of PDIp function.

The physiologic role of PDIp in the brain is not known. PDIp is highly expressed in the acinar cells of the pancreas [7]. PDIp expression in both pancreatic acinar cells and in dopaminergic neurons may reflect the largely secretory function of both types of cells. Pancreatic acinar cells secrete digestive enzyme zymogens, and dopaminergic neurons secrete DA in response to hormonal and neural signals (regulated secretion); which differs from the continuous movement of proteins to the cell surface in transport vesicles (constitutive secretion) [2]. This suggests the possibility that in DA neurons PDI may facilitate the folding of constitutively secreted proteins and PDIp may facilitate the folding of regulated secreted proteins in the ER.

The connection between PDIp expression and proteasome dysfunction is also unknown. One possibility is that PDIp functions as chaperone in the unfolding of protein substrates, such as tyrosine hydroxylase, destined for degradation by the proteasome [8]. The 26S proteasome is relatively ineffective at degrading aggregated proteins [20] and requires that substrates be unfolded to gain access to the active site and. This would require that PDIp have access to the cytoplasm. The subcellular distribution of PDIp in DA neurons remains to be determined; however, at least two other members of the PDI family, PDI and ERp57 are found in the cytosol [34]. It is also possible that PDIp exists in the ER as part of a complex containing both ER membrane and cytosolic components involved in degrading misfolded ER proteins. Data indicating that PDI is involved in such a complex have recently been published [27,34].

In summary, we show that the PDI family member PDIp is uniquely upregulated among family members within 24 h following exposure of differentiated SH-SY5Y cells to 1 mM MPP^+. Comparisons of PDIp expression among cultures treated with lactacystin suggest that impaired proteasome function may be important mechanistically in the up-regulation of PDIp. Gene expression and immunohistochemical studies using postmortem brain tissue demonstrate that PDIp is expressed in the brain and that PDIp immunoreactivity is associated with LB pathology. Collectively, these findings suggest that increased PDIp expression in proteasome impaired DA neurons may participate in LB formation and perhaps contribute to neurodegeneration. The identification of the structural components of the inclusions found in the brains of individuals afflicted with neurodegenerative diseases provides insight into the pathological mechanism involved. The identification of the PDI family member, PDIp, may provide a new understanding of the pathological mechanism involved in LB formation.

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Upregulation of Clusterin/apolipoprotein J in Lactacystin-treated SH-SY5Y Cells

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ABSTRACT:

Clusterin (apolipoprotein J) is a highly conserved, multifunctional, vertebrate glycoprotein. Several isoforms of clusterin have been described including the predominant secreted isoform (sCLU) and several nuclear isoforms (nCLU) associated with cell death. sCLU has been shown to bind a variety of partly unfolded, stressed proteins including those associated with Lewy bodies (LBs) in patients with Parkinson's disease (PD). The development of familial and sporadic PD has been associated with the ubiquitin-proteasome system (UPS) dysfunction and aberrant protein degradation. This suggests that failure of the UPS to degrade abnormal proteins may underlie nigral degeneration and LB formation in PD. To explore this concept, the effects of toxin-mediated proteasomal impairment on changes in gene expression and cell viability were studied in differentiated SH-SY5Y cells. Clusterin expression was increased in cells exposed for 24 hours to the proteasomal inhibitor lactacystin (10 μM) as determined by gene microarray analysis. RT-PCR showed that sCLU, not nCLU, was the major clusterin isoform expressed in both control and lactacystin treated cells. Western blot analysis identified statistically significant increases in sCLU in total cell lysates after 24 hours of lactacystin exposure and showed that sCLU fractionates with the endoplasmic reticulum. Time course studies demonstrated that maximal decreases (4 hours) in proteasome activity preceded maximal increases (24 hours) in clusterin expression. Together these data suggest that proteasome impairment contributes significantly to the upregulation of sCLU in SH-SY5Y cells supporting the hypothesis that the association of clusterin with LBs in PD may be related to UPS failure.

KEY WORDS: Parkinson's disease, Proteasome, Gene expression

INTRODUCTION:

Clusterin is a highly conserved, nearly ubiquitous, vertebrate protein known by numerous names including apolipoprotein J (ApoJ) (Jones and Jomary, 2002). The expression of the clusterin gene (CLU) is complex, resulting in different clusterin isoforms that appear in different cell compartments. Translated as a 449 amino acid precursor, clusterin possesses a signal peptide that guides it into the endoplasmic reticulum (ER) and is glycosylated and internally cleaved before it is secreted. The secreted form of the clusterin protein (sCLU) is a heterodimeric sulfated 80 kDa glycoprotein that appears as a ~40 kDa α- and β-protein smear by SDS-PAGE under reducing conditions (Yang et al., 1999). sCLU possesses nonspecific binding activity to hydrophobic domains of various proteins *in vitro* (Humphreys et al., 1999) and has been hypothesized to function as a molecular chaperone (Bailey et al., 2001; Clark and Griswold, 1997; Kimura et al., 1997; Michel et al., 1997).

In addition to sCLU, several intracellular clusterin isoforms have been described (Debure et al., 2003; Leskov et al., 2003; Reddy et al., 1996; Senut et al., 1992). These include several nuclear isoforms associated with cell death. For example, a 42-kDa nuclear glyco/isoform of clusterin is up-regulated in epithelial cells undergoing apoptosis (Lakins et al., 1998). Similarly, a 45 kDa isoform of clusterin has been described which, when targeted to the nucleus, can decrease cell proliferation and promote cell detachment-induced apoptosis in human prostate epithelial cells (Caccamo et al., 2003; Caccamo et al., 2004). Lastly, it has been shown that a spliced variant of sCLU, lacking exon II, gives rise to a nuclear form of clusterin (nCLU) that is targeted to the nucleus

following ionizing radiation (Leskov et al., 2003) and that overexpression of nCLU results in caspase-3-independent apoptosis (Yang et al., 2000).

Besides being associated with cell death, clusterin has been related to numerous other biological processes including cytoprotection, sperm maturation, lipid transport, regulation of the complement cascade, membrane recycling, immune regulation, cell adhesion, and morphological transformation (Rosenberg and Silkensen, 1995). Clusterin gene transcription is responsive to cellular stress (Michel et al., 1997) and is induced after tissue injury and in many disease states such as glomerulonephritis, polycystic kidney disease, renal tubular injury, atherosclerosis, myocardial infarction and neurodegenerative conditions including Alzheimer's disease (AD) where it associated with amyloid plaques (Choi-Miura et al., 1992; Lidstrom et al., 1998; May and Finch, 1992; McGeer et al., 1992; Oda et al., 1994; Rosenberg and Silkensen, 1995). In addition, clusterin has been found in glial cytoplasmic inclusions (GCIs) in cases with multiple system atrophy (MSA) and is associated with Lewy bodies in patients with dementia with Lewy bodies (DLB) and Parkinson's disease (PD) (Sasaki et al., 2002).

The pathological hallmarks of PD are the degeneration of dopamine (DA)-containing neurons in the substantia nigra (SN) and the presence of Lewy bodies (LBs) in many of the surviving DA neurons (Forno, 1996). The majority of PD cases are sporadic. The identification of a number of genes associated with rare familial forms of PD, however, has provided important insights into the underlying mechanisms of the disease. These genes, encoding α-synuclein, parkin, UCH-L1, DJ-1, and PINK1 have implicated protein misfolding, oxidative stress, and impairment of the ubiquitin-proteasome system (UPS), in the pathogenesis of the disease (Dauer and Przedborski, 2003; Dawson and

Dawson, 2003; Halliwell, 2002; McNaught et al., 2001; Valente et al., 2004). The ubiquitin-proteasome system (UPS) is the primary biochemical pathway responsible for the degradation of normal and abnormal intracellular proteins (Hershko and Ciechanover, 1998; Hochstrasser, 1996). Proteolysis by the proteasome is specifically inhibited by the *Streptomyces* metabolite lactacystin (Fenteany and Schreiber, 1998). UPS failure leads to protein accumulation, cell death (Sherman and Goldberg, 2001; Voges et al., 1999) and possibly LB formation (Alves-Rodrigues et al., 1998; Andersen, 2000; Carrell and Lomas, 1997; Dickson, 2001).

MATERIALS AND METHODS:

Materials- Lactacystin, Dulbecco's modified Eagle's Medium (DMEM), retinoic acid (RA), Tri-reagent (Sigma-Aldrich; St. Louis, MO), and fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) were purchased from commercial sources.

Cell culture, differentiation, and toxin treatment - The undifferentiated human neuroblastoma cell line SH-SY5Y (ATCC CRL-2266) was cultured and differentiated with RA as described previously (Conn et al., 2003). Briefly, undifferentiated cells were maintained in DMEM-high glucose supplemented with 10% FBS. For toxin experiments 0.8 x 10^6 cells were plated into 100 mm^2 dishes (Corning; Cambridge, MA) in differentiation media (10 ml DMEM plus 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin and 10 μM RA) and cultured for 6 days, with one media change, prior to

the addition of freshly prepared lactacystin (10 μM) or vehicle, phosphate buffered saline (PBS), for various lengths of time in fresh differentiation media.

Microarray analysis- 15 x 10^6 cells were treated for 24 hours with lactacystin (10 μM) or control conditions (vehicle) prior to being harvested for custom gene microarray analysis using the plastic human 12K microarray (BD Biosciences; Mt. View, CA).

RNA isolation and reverse transcription – RNA isolation and reverse transcription was performed as previously described (Conn et al., 2001). Briefly, total RNA was isolated from each experimental condition using Tri reagent (Sigma-Aldrich; St. Louis, MO) and treated with 1 unit DNAse-I using the MessageClean kit (GenHunter Corp; Nashville, TN). DNAse-treated RNA was subjected to PCR analyses, as described below, using primers to G3PDH to ensure that each RNA preparation was free of DNA. DNAse-treated RNA (5 μg) was reverse transcribed using oligo(dT) primers provided in the Superscript kit (Clonetech; Palo Alto, CA).

PCR amplification, visualization and quantification – PCR reactions for the amplification of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; G3PDH) were performed as described previously (Conn et al., 2001). Clusterin expression was detected using a primer set previously shown to distinguish sCLU from nCLU (Leskov et al., 2003) and with primers designed (Rozen and Skaletsky, 2000) to clusterin (gi20455818) corresponding to base pairs (bp) 2417 to 2436 (5' TCACTGAGGTGGTCGTGAAG 3') and 2661 and 2642 (5' TACTTGGTGACGTGCAGAGC 3'). PCR conditions were

optimized to give products in the linear range of detection using the 4400 ChemiImager low-light imaging system (Alpha-Innotech, San Leandro, CA): 30 cycles of 95 °C for 30 seconds, 68 °C for 45 seconds, and 72 °C for 45 seconds using the primers described by Leskov *et al* and 30 cycles of 94 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 2 minutes using the primers described above.

Subcellular fractionation- Enriched nuclear, endoplasmic reticular, and soluble cytosolic fractions were isolated as described previously (Rao et al., 2001). Briefly, cells were harvested from 100 mm^2 dishes in ice-cold fractionation buffer, pH 7.4 (20 mM HEPES, 10 mM KCl, 250 mM sucrose, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 X protease inhibitor cocktail) and homogenized on ice with 25 passes of a 2-ml Kontes Dounce Tissue Grinder (Fisher Scientific; Rochester, NY). After cell lysis, nuclei were pelleted by centrifugation for 10 minutes (750 $\times g$ at 4 °C) and resuspended in nuclear lysis buffer, pH 7.5 (20 mM Tris, 420 mM KCl, 1.5 mM MgCl2, 20% glycerol, 0.5% triton X100, and 1 X protease inhibitor cocktail). The supernatant was collected from the nuclear pellet and clarified by centrifugation for 30 minutes (10,000 $\times g$ at 4 °C). The resultant supernatant was collected and centrifuged for 60 minutes (100,000 $\times g$ at 4 °C) to obtain an ER pellet and a soluble cytoplasmic supernatant. The ER pellet was lysed in ER buffer, pH 7.4 (10 mM Tris 0.5% triton X100 and 1 X protease inhibitor cocktail). The protein concentrations of the enriched fractions were determined using the BCA kit (Pierce; Rockford, IL) and equal amounts of protein from each fraction were subject to Western blot analysis as described below.

Western blot analysis- Western blot analysis was performed as described previously (Conn et al., 2001). Briefly, cells were harvested in lysis buffer (20 mM Tris pH 7.4, 140 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 X protease inhibitor cocktail) and 50 µg of protein from each experimental condition were resolved by SDS PAGE and electrophoretically transferred to nitrocellulose membranes. After blocking membranes with 5% non-fat milk, membranes were incubated with either a 1µg/ml dilution of polyclonal goat anti-apolipoprotein J (Chemicion International; Temecula, CA) or a 1:1,000 dilution of polyclonal rabbit anti-PDI (Stressgen Biotechnologies; Victoria, BC Canada). After washing, membranes were incubated with 1:10,000 dilution of donkey anti-goat or goat anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (Chemicon International Inc; Temecula, CA), respectively. Membranes were washed prior to incubation with SuperSignal West Pico Chemiluminescent Substrate (Pierce; Rockland, IL). Membranes were visualized with the 4400 ChemiImager low light imaging system (Alpha-Innotech; San Leandro, CA). Exposure times were optimized to collect images of protein products within the linear range of detection for the Chemiimaging system.

20S Proteasome Activity Assay- Cells were harvested from 100 mm^2 dishes in 300 µl of PBS using a plastic cell lifter. Harvested cells were pelleted by centrifugation (12,000 x g at 4 °C), resuspended in 100 µl lysis buffer (PBS with 1% Triton X-100) and frozen to -70 °C. Thawed cell suspensions were sonicated using a Branson sonifier 450 (30 seconds with a 50% duty cycle at a power level of 20 W) and clarified by centrifugation (12,000 x g at 4 °C). The protein concentrations of the resultant supernatants was determined using

the BCA kit (Pierce; Rockford, IL). The supernatant was assayed for proteasome activity using the 20S Proteasome Activity Assay Kit (Chemicon; Temecula, CA) according to the manufacturer's instructions. Briefly, 20 µg of protein from control (con) and each experimental (exp) condition was incubated with (+) and without (-) 3.125 µM lactacystin (lac) for 10 minutes at room temerature prior to being incubated with 5 µM of the LLVY-7-amino-4-methylcoumarin (AMC) flourogenic substrate at 60 °C for 60 minutes. Flourescence was quantified as relative fluorescence units (RFU) using a 355/460 nm filter set in a microtiter plate flourometer MFX (Dynex Technologies; Chentilly, VA) Proteasome activity, expressed as % control, was caluculated as: $(RFU_{exp - lac} - RFU_{exp + lac} / RFU_{con - lac} - RFU_{con + lac}) \times 100$.

Cell viability assay- The MTT assay was used to evaluate cell viability. Cells were plated at a density of 1×10^4 cells/well in 48-well tissue culture plates in 500 µl of differentiation media. Cells were cultured for various lengths of time and the conversion of soluble 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to insoluble formazan by mitochondrial dehydrogenase activity was assayed using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega; Madison, WI).

Statistical Analysis- Values represent the means ± SEM. Differences among means were analyzed by student's t-test or one-way ANOVA with time or treatment as the independent factor. When ANOVA showed significant differences, post-hoc tests were performed.

RESULTS:

To explore the hypothesis that UPS failure may underlie nigral degeneration and LB formation in PD, we studied the effects of toxin-mediated proteasomal impairment on changes in gene expression and cell viability. RA-differentiated SH-SY5Y cells were treated for 24 hours with lactacystin (10 μM) or control conditions (vehicle) prior to being harvested for custom gene microarray analysis using the plastic human 12K microarray (BD Biosciences; Mt. View, CA). Of the 683 genes identified by the manufacturer as differentially expressed, the clusterin gene showed the greatest change (Figure 1).

To confirm the gene microarray results RT-PCR analyses were performed using RNA isolated from cells treated with and without 10 μM lactacystin for 0.5, 2, 4, 8, 16, and 24 hours and primers corresponding to bp 2417 to 2436 and 2661 and 2642 of the clusterin gene (gi20455818). Parallel PCR reactions were also performed using primers to the housekeeping gene G3PDH to determine the specificity of the gene expression response. The results from a typical RT-PCR experiment using primers to clusterin and G3PDH (Figure 2A) show that lactacystin does not alter the expression of G3PDH. In contrast, clusterin gene expression was increased in lactacystin-treated cells as compared to control cells at 8, 16 and 24 hours.

To study what isoform(s) of clusterin were expressed in control and lactacystin-treated cells, RT-PCR analysis was performed using RNA isolated from cells treated with and without 10 μM lactacystin for 24 hours and primers to clusterin previously shown to amplify both the full length secreted form of clusterin, sCLU, and the alternatively

spliced, cell-death, nuclear isoform of clusterin, nCLU (Leskov et al., 2003). The results from a typical RT-PCR experiment (Figure 2B) show that a single PCR product was detected using RNA from both control and lactacystin-treated cells corresponding to the expected size of the sCLU isoform.

To determine apparent molecular mass of the clusterin isoform(s) expressed in control and lactacystin treated SH-SY5Y cells and to determine the influence of lactacystin on steady-state clusterin protein levels, Western blot analyses were performed using total cell lysates isolated 24 hours after exposure of cells to lactacystin (10 μM) or control conditions and polyclonal antibodies to recombinant sCLU. The results from a typical Western blot experiment (Figure 2C) show that the major clusterin polypeptide in both control and lactacystin treated cells migrates with an apparent molecular mass of ~40 to 45 kDa and that steady-state clusterin protein levels increase in SH-SY5Y cells following lactacystin exposure. Quantification of steady-state clusterin protein levels from four independent experiments demonstrated that the increase in clusterin in lactacystin-treated cultures after 24 hours is 124.7% ± 6.67% (mean ± SEM, one sample t-test, $p<0.05$).

The subcellular localization of clusterin in cells exposed for 24 hours to control or lactacystin (10 μM) was examined by Western blot using enriched nuclear, ER, and soluble cytosolic fractions and antibodies to clusterin. The result from a typical Western blot experiment (Figure 2D) show that the major clusterin polypeptide in both control and lactacystin treated cells fractionates with the ER. No clusterin was found in the nuclear or soluble cytosol fractions. The integrity of the fractions was monitored in parallel Western

blot experiments using antibodies to the ER resident protein disulfide isomerase and by Coomassie staining of gels after transfer (data not shown).

The temporal relationship between clusterin gene expression, 20S proteasome activity, and cell viability was evaluated in RA-differentiated SH-SY5Y cells treated with and without 10 μM lactacystin for 0.5, 2, 4, 8, 16, 24, and 48 hours (Figure 3). Maximal decreases in proteasome activity were detected 4 hours after lactacystin exposure (38.7 % ± 3.92%; mean ± SEM, two-tailed one sample t-test $p<0.0001$) and preceded maximal increases in clustering gene expression at 24 hours (193.1% ± 18.2%; mean ± SEM, one sample t-test, $p<0.005$). Maximal changes in clusterin gene expression and proteasomal activity both preceded decreases in cell viability as measured by the MTT which is not detected until 24 hours after lactacystin exposure (76.3% ± 3.38%; mean ± SEM, two-tailed one sample t-test $p<0.0005$).

DISCUSSION:

The results of this study indicate that proteasome impairment contributes significantly to the upregulation of clusterin by lactacystin. This is of particular interest in furthering our understanding of the pathogenesis of PD as it suggests that the association of clusterin with LBs in PD (Sasaki et al., 2002) may be related to UPS failure. There is increasing evidence that UPS failure may underlie nigral degeneration and LB formation in both sporadic and familial PD (Dauer and Przedborski, 2003; Dawson and Dawson, 2003; Halliwell, 2002; McNaught et al., 2001; Valente et al., 2004). Recently, it has been shown that synthetic (Z-Ile-Glu(O*t*Bu)-Ala-Leu-al; PSI) and naturally occurring

(epoxomicin) inhibitors of proteasomal function can induce a progressive disorder in rats that closely recapitulates the features of PD (St et al., 2004). Our finding relating proteasomal impairment to increased clusterin expression is supported by the observation that in WI38 fibroblasts, epoxomicin exposure increases both sCLU and a cytoplasmic 64 kDa isoform of clusterin (Chondrogianni et al., 2003).

Increased clusterin expression has been associated with cell death responses in various tissues after toxic stress signals (Ahuja et al., 1994; Bursch et al., 1995; Buttyan et al., 1989; Danik et al., 1991; May, 1993; Sensibar et al., 1995; Tenniswood et al., 1992; Wong, 1994). Increased clusterin expression has been reported to be both cytoprotective (Criswell et al., 2003; Michel et al., 1997; Popper et al., 1997) and cytotoxic with a number of laboratories showing that cell death forms of clusterin are targeted to the nucleus (Caccamo et al., 2003; Caccamo et al., 2004; Lakins et al., 1998; Leskov et al., 2003; Yang et al., 2000).

There is no evidence that clusterin functions as an inducer of cell death in SH-SY5Y cells following lactacystin exposure. By RT-PCR, sCLU but not nCLU was detected in both control and lactacystin-treated cells. Western blot analysis following subcellular fractionation also failed to detect clusterin associated with the nucleus. The apparent molecular mass of clusterin (~40-45 kDa) and the association of clusterin with the ER suggest that in both control and lactacystin-treated cells sCLU is the major isoform expressed.

There is a growing body of evidence to suggest that stress-increased expression of sCLU is a cytoprotective effort (Criswell et al., 2003) and that sCLU exerts cytoprotection by functioning as a protein chaperone (Humphreys et al., 1999). sCLU is

capable of binding a wide range of partners (Wilson and Easterbrook-Smith, 2000) including partially folded stressed proteins (DeMattos et al., 2002; Poon et al., 2002) such as the major LB polypeptide component, α-synuclein (Zhou et al., 2004). The intracellular localization of LBs and the observation that *in vitro* astrocytes, but not neurons, secrete clusterin (Morgan et al., 1995; Pasinetti et al., 1994) suggest that in neurons clusterin exert its biological function intracellularly. Our finding that clusterin localizes to the ER, suggests that in neurons clusterin may possibly function as a stress-inducible ER protein chaperone.

It remains unclear how clusterin becomes associated with LBs. LB formation has been hypothesized to an aggresome-related event in response to increasing levels of abnomal proteins in neurons (McNaught et al., 2002). Aggresomes are structures that accumulate misfolded proteins thereby protecting against the deleterious spreading of misfolded proteins in the cell (Chung et al., 2001). Recently, it has been found that clusterin has strong aggresome forming capacity (Debure et al., 2003).

One possible model of the relationship between UPS failure, clusterin gene expression, LB formation and neurodegeneration in PD (Figure 4) is that in DA neurons clusterin expression is increased as a result of UPS failure caused by genetic and/or environmental insults. Functioning as a protective chaperone molecule, clusterin becomes associated with misfolded/damaged proteins that accumulate as a result of UPS failure thereby promoting cell survival. Over time, clusterin bound to its substrate becomes sequestered in within aggresomes and later LBs leading essentially to a loss of protective chaperone function and cell death.

In conclusion, the results of this study suggest that proteasome impairment contributes significantly to the upregulation of sCLU in SH-SY5Y cells supporting the hypothesis that the association of clusterin with LBs in PD may be related to UPS failure.

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FIGURE LEGENDS:

Figure One- *Gene microarray analysis of lactacystin toxicity*- Shown are of images of plastic human 12K microarrays generated from cells treated for 24 hours with lactacystin (10 μM) or control conditions (vehicle). The arrows show differential gene expression of clusterin.

Figure Two- *RT-PCR and Western blot analyses of clusterin expression*- Cells were exposed to 10 μM lactacystin (L) or control (C) conditions for up to 24 hours prior to being harvested for RT-PCR and Western blot analyses. **A.** Electrophoretic separation of PCR products from a typical experiment is shown using RNA isolated from cells after incubation with (+) or without (-) lactacystin for 0.5, 2, 4, 8 16, and 24 hours and primers to G3PDH and clusterin **B**. Electrophoretic separation of PCR products from a typical

experiment is shown using RNA isolated 24 hours after exposure of cells to lactacystin (L) or control (C) conditions and primers to clusterin capable of discriminating sCLU (340 bp) from the alternatively spliced nuclear nCLU (220 bp) isoform. A 50 bp DNA ladder is shown to the left. **C.** Western blot analysis from a typical experiment is shown using total cell lysates isolated 24 hours after exposure of cells to lactacystin (L) or control (C) conditions and antibodies to clusterin (left panel). Coomassie staining of the gel after transfer (right panel) was performed to monitor equal loading of protein. Molecular weight standards in kilodaltons (kDa) are shown to the left of the blot. **D.** Western blot analysis was performed on enriched nuclear (N), endoplasmic reticular (ER) and soluble cytosol (S) fractions obtained from control (C) and lactacystin-treated (L) cells with antibodies to clusterin.

Figure Three - *Timecourse of clusterin gene expression, proteasomal activity, and cell viability in SH-SY5Y cells after exposure to 10 μM lactacystin-* Cells were exposed to lactacystin (L) or control conditions (C) for 0.5, 2, 4, 8, 16, 24, and 48 hours. At each timepoint, clusterin and G3PDH gene expression, cell viability, proteasomal activity, was quantified as described in the text. Data are graphed as % control where control represents those cells not treated with lactacystin. Time course represents the mean of quadruplicate values from a single experiment except for values with error bars where the data represents the mean ± SEM from 4 to 7 independent experiments.

Figure Four – *Model of the relationship between UPS failure, clusterin gene expression, LB formation and neurodegeneration in PD-* Clusterin expression is increased in DA

neurons as a result of UPS failure caused by genetic and/or environmental insults. Functioning as a protective chaperone molecule, clusterin becomes associated with misfolded/damaged proteins that accumulate as a result of UPS failure thereby promoting cell survival. Over time, clusterin bound to its substrate becomes sequestered in within aggresomes and later LBs leading essentially to a loss of protective chaperone function and cell death.

FIGURE ONE

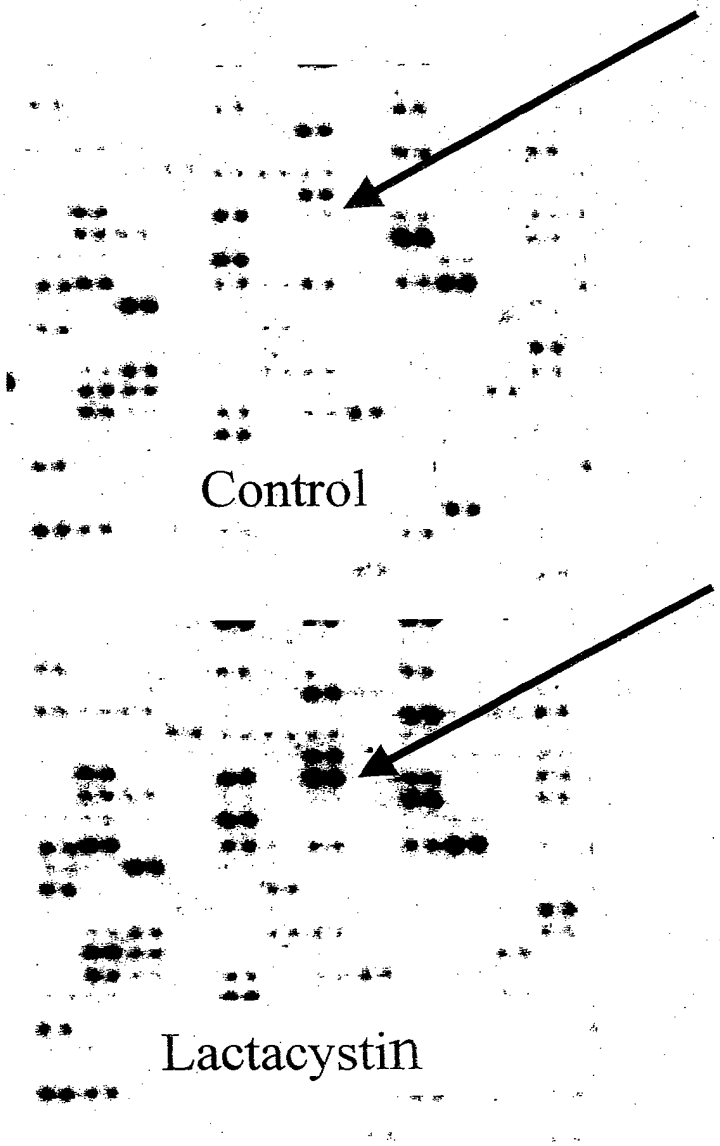
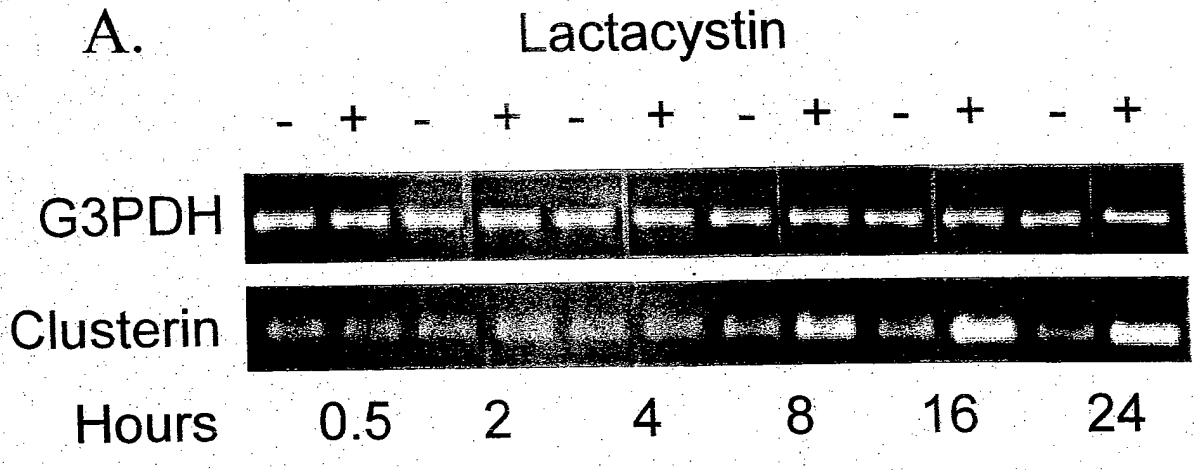
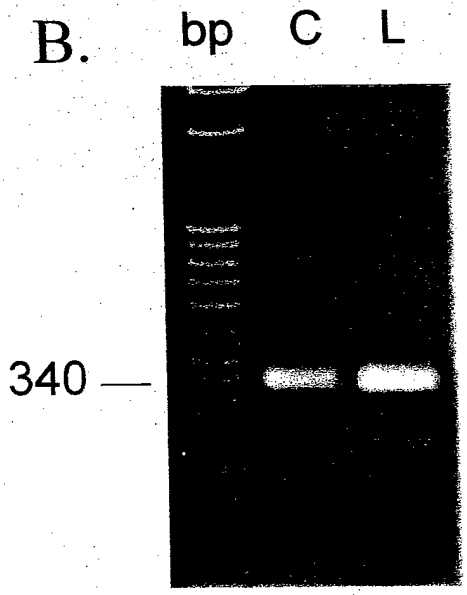
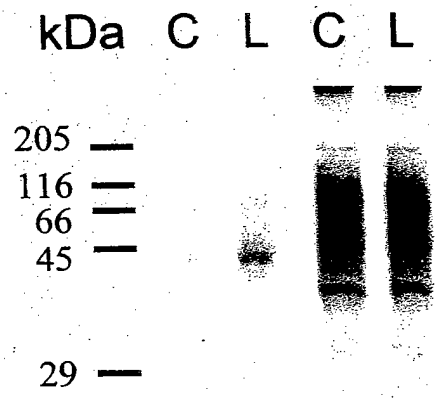


FIGURE TWO







clusterin gel

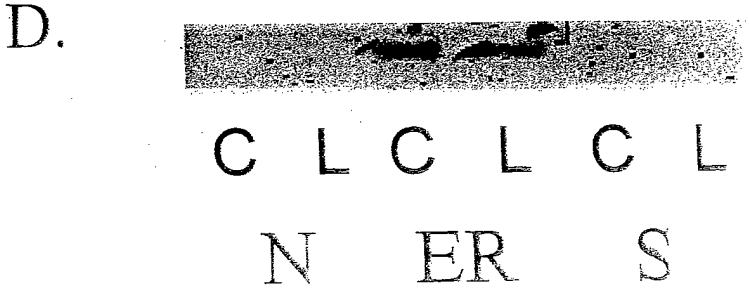


FIGURE THREE

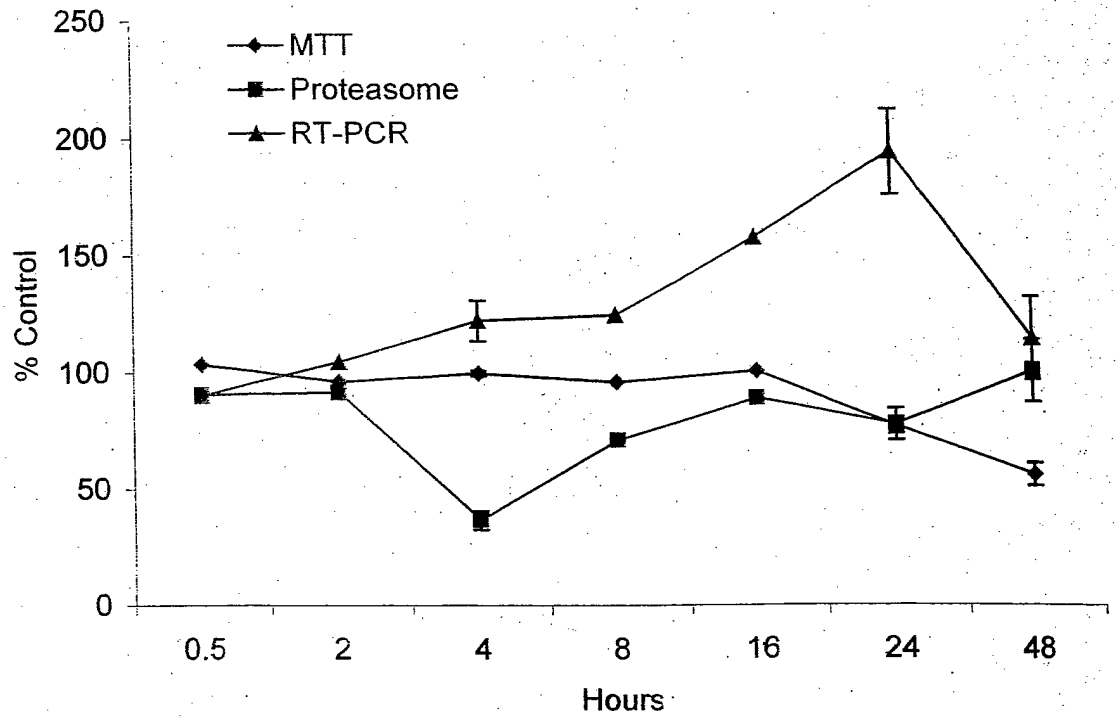
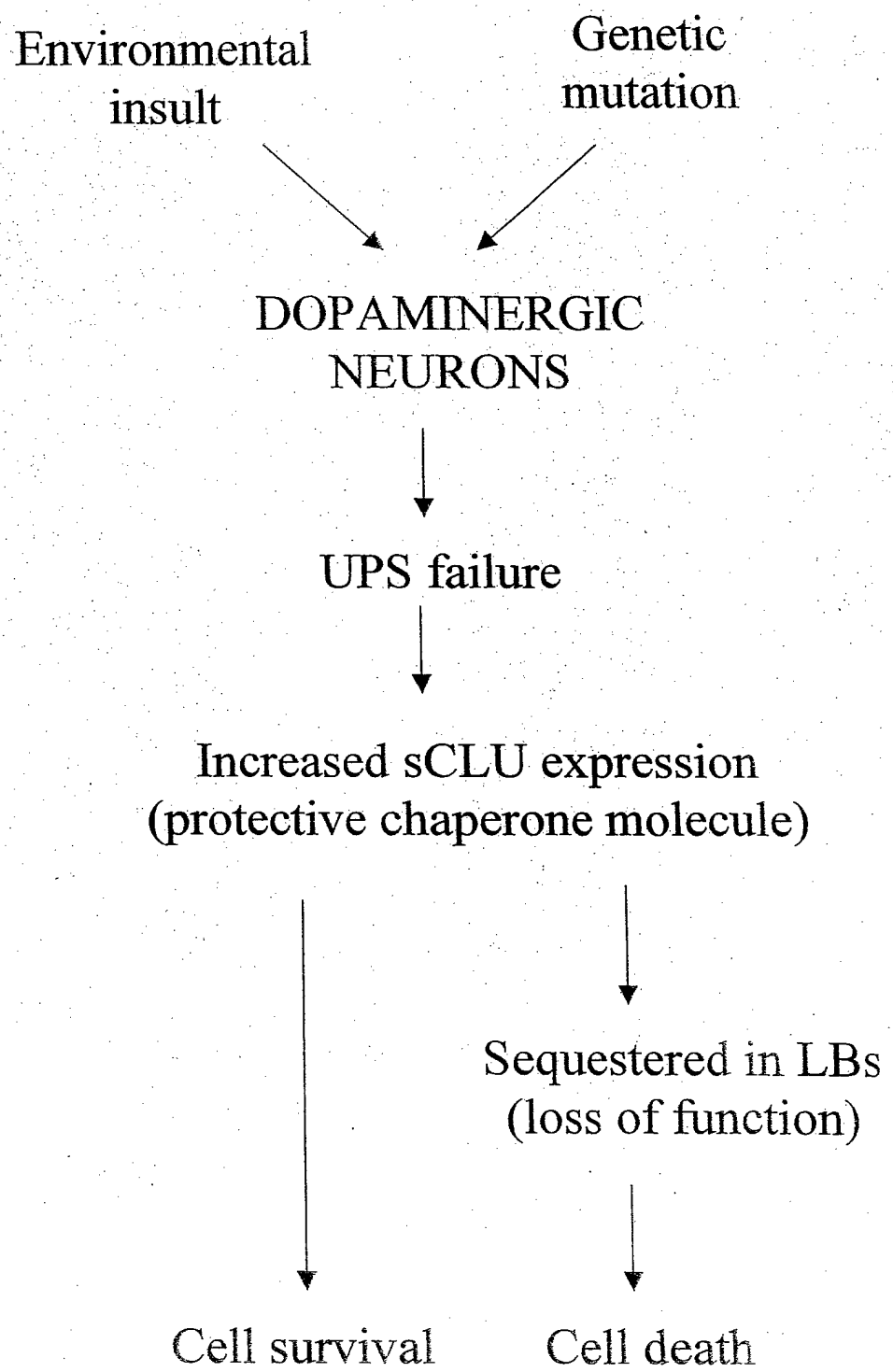


FIGURE FOUR


Environmental insult
Genetic mutation
DOPAMINERGIC NEURONS
UPS failure
Increased sCLU expression (protective chaperone molecule)
Sequestered in LBs (loss of function)
Cell survival
Cell death